REVIEW ARTICLE

p53 tumor suppressor gene therapy for cancer

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The last two decades have led to a greater understanding of the genetic basis of human malignancy. Although numerous genetic alterations have been detected in cancer, activation of oncogenes and inactivation of cell cycle regulators (e.g., tumor suppressor genes) are now known to play a critical role in the progression of the disease. Therapeutic strategies based on specific molecular alterations in cancer include reintroduction of wild-type tumor suppressor function to cells lacking the gene. p53 gene therapy provides an attractive strategy to test the potential clinical feasibility of this approach. Alterations in p53 function are present in approximately half of all malignancies, and expression of wild-type p53 can result in apoptosis in human tumor cells. This review summarizes current investigations with p53 gene therapy, highlighting the preclinical efforts with adenoviral, retroviral, and lipid-based gene delivery systems. A comprehensive review of the various clinical targets suggested for p53 gene therapy is presented together with challenges and prospects for future clinical investigation.

Key words: p53 gene therapy; p53 tumor suppressor gene; adenovirus; retrovirus; liposomes; gene therapy vectors; review article.

The p53 tumor suppressor is a 393-amino acid nuclear phosphoprotein that acts as a transcription factor to control the expression of proteins involved in the cell cycle.[1,2] In response to DNA damage, wild-type p53 accumulates in the nucleus and arrests the cell cycle via the cyclin-dependent kinase inhibitor p21WAF1/CIP1. Alternatively, p53 can induce apoptosis or programmed cell death through both transcription dependent (e.g., bax, Fas) and transcription-independent pathways. Because of these functions, p53 has been called the "guardian of the genome" and loss of p53 has been implicated in tumor progression. Functional inactivation of p53 can occur by several mechanisms including direct genetic mutation, binding to viral oncoproteins or cellular factors (e.g., mdm2), or alteration of the subcellular localization of the protein. Although p53 is not essential for normal development, p53 "knock-out" mice are susceptible to tumors early in life. Mutations in p53 have been reported in a majority of clinical cancers, and it has been estimated that p53 function is altered in half of all human malignancies. Non-random mutations in p53 have been reported in clinical specimens, and frequent mutations correspond to evolutionarily conserved regions of the molecule. Of particular significance, alterations in p53 are linked to poor prognosis, disease progression, and decreased sensitivity to chemotherapeutic agents. Detailed reviews on p53 function have appeared recently,[3–5] and the reader is directed to them for a more detailed description of p53 structure and function.

Consistent with the definition of a tumor suppressor gene, reintroduction of the wild-type p53 has been shown to be incompatible with the tumorigenic phenotype of many tumor cell lines. Early experimental work with neoplastic cells stably transduced with wild-type p53 demonstrated a suppression of cell growth, decrease in colony formation, and reduction in tumorigenicity in nude mice.[6,7] In addition, Shaw et al[8] provided evidence that re-expression of p53 in established tumors can induce apoptosis *in vivo*. More recent efforts, summarized below, have confirmed these initial findings with gene therapy vectors suitable for human clinical trials. Results from preclinical studies also suggest that nontransformed cells can tolerate exogenous expression of p53, providing a potential therapeutic index for the treatment of cancer.

GENE DELIVERY SYSTEMS

Clinical investigations using gene therapy have only recently been initiated, and many obstacles to efficient gene delivery have been identified. Successful gene therapy strategies will match a gene delivery system with a gene for a particular clinical application.[9] Although the p53 gene is altered in many human cancers, a single gene delivery system is not likely to be optimal for all indications. For example, intratumoral delivery to p53-altered head and neck tumors may require high local concentrations of a viral vector, whereas an alternative gene delivery system may be required to target blood-borne or metastatic disease. In addition, the requirement for integration of a transgene will govern the selection of a

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gene delivery system. Because overexpression of high levels of p53 can trigger apoptosis in tumor cells, gene delivery methods that result in transgene integration into the host genome are probably not necessary, or even desirable, for successful p53 gene therapy. The sections below summarize some of the advantages and disadvantages associated with the gene delivery systems currently in use. For detailed reviews on vector construction, the reader is referred to Jolly.[10]

Retroviruses

Replication-deficient retroviruses were the first viral vectors used in gene therapy and can accommodate approximately 8 kb of foreign DNA. Although these vectors have advantages for treating hereditary disorders, there are several disadvantages associated with retrovirus delivery of the p53 gene. Because retroviruses integrate genetic material into the host cell genome, both tumor and normal cells can be permanently modified. Although normal cells appear to tolerate the expression of exogenous wild-type p53, the potential for insertional mutagenesis raises safety concerns for retrovirus-based *in vivo* gene therapy. Retroviruses require cell division for efficient infection and transfer of new genetic information. Therefore, preferential transduction of rapidly dividing tumor cells can be expected with retroviruses. In addition, retroviral vectors unlike other viral gene delivery systems do not retain viral genes that may invoke an immune response. However, poor stability and low titers from current production processes are likely to limit the use of replication-deficient retroviruses to *ex vivo* gene therapy. Developments with retrovirus-based gene delivery have recently been reported,[11] but future improvements of these vectors are required to fully enable *in vivo* gene delivery of p53.

Adenoviruses

Alternative viral gene delivery systems have been explored for gene therapy, including those based on adenovirus, adeno-associated virus, herpes virus, and vaccinia virus. Among these, adenoviral vectors have received the greatest attention and have been used in clinical studies for cancer and cystic fibrosis.[9] Significant advantages of adenoviral vectors include 1) the ability to transduce both proliferating and quiescent cells, 2) a wide tissue tropism, and 3) the existence of efficient protocols for producing clinical-grade material at high concentrations. In contrast to retroviruses-mediated gene transfer, adenoviral DNA remains extrachromosomal, and integration into the host cell genome is not an obligate part of the viral life cycle. Consequently, transient transgene expression can be expected with replication-deficient, recombinant adenoviruses. Short term expression has obvious limitations for chronic gene replacement, but may be considered advantageous for the treatment of neoplasias. For example, transient overexpression of p53 in tumor tissue may be sufficient to activate the apoptotic pathway in neoplastic cells without the concerns associated with integration of genetic material into normal cells. Several investigators have reported the antitumor effects of adenoviruses encoding wild-type p53 in various preclinical models (see below).

Replication deficient adenoviruses have only recently been used in clinical studies. Severe adverse effects have not been reported following administration to patients with cystic fibrosis or cancer.[9] Interestingly, one published clinical study has reported the use of wild-type adenovirus when administered via the following routes: intratumoral, intra-arterial, and/or intravenous.[12] Thirty patients with advanced cervical cancer were dosed with no serious side-effects. Tumor necrosis was observed, but no pathological effects on vaginal, rectal, or bladder mucosa, or other tissues of the pelvis were found. There was no evidence of lesions in the central nervous system, liver, or other organs attributable to the adenovirus therapy.

Of particular concern with adenoviral vectors is the induction of a cellular and humoral immune response in treated patients. The nature and extent of the immune response to recombinant adenovirus has not been fully characterized in humans, but preclinical models suggest that neutralizing antibodies directed at the adenovirus capsid proteins may compromise gene transfer after repeat administration. A cytotoxic T-lymphocyte (CTL) response has also been reported after administration of recombinant adenoviruses. Although the relative contributions of the transgene and viral gene expression are currently unclear in this process, the host immune response leads to elimination of transduced cells. While the CTL response may provide an additional antitumor effect, the potential for toxicity to normal cells requires evaluation in both preclinical and clinical models.

Delivery of wild-type p53 via an adenoviral vector is likely to provide the first demonstration of effective p53 gene therapy in the clinic for a variety of human cancers. Because local administration is practical, initial clinical trials include intratumoral, intra-arterial (hepatic), intraperitoneal, and intracystic (bladder) delivery. Dosing strategies to overcome the expected immune response include transient immunosuppression to facilitate multiple administration as well as construction of "second generation" recombinant adenoviruses with limited expression of immunogenic, adenovirus late genes. Ongoing clinical trials will provide critical information on the dose and route dependence of the immune response to recombinant adenoviruses.

Nonviral gene delivery

Complexation of cationic lipids with DNA provides an alternative to viral gene delivery systems. Although less efficient, this lipid-based gene delivery system is likely to be less toxic and less immunogenic than strategies that use recombinant viruses. Both viral and nonviral gene delivery systems provide an opportunity to demonstrate the effects of p53 gene therapy on locoregional malignancies. However, delivery of the p53 gene to metastatic tumors via systemic administration will likely require

multiple administrations of a nonimmunogenic (i.e., nonviral) gene delivery system. The challenges associated with systemic delivery of macromolecules to tumors has been well documented.[13] Despite these obstacles, gene transfer to both normal and neoplastic tissue has been reported after lipid-based gene delivery.[14] Although the relative efficiency of transgene expression is low compared with viral vectors, systemic delivery of p53 has been shown to inhibit tumor growth in animal models. These studies suggest that the efficacy of p53 gene therapy may be due to broader mechanisms than originally suspected, i.e., inhibition of angiogenesis and other "bystander" effects (see below). Additional improvements in the efficiency of nonviral gene delivery systems are expected, and such improvements may enable systemic delivery that can be enhanced by tumor targeting.

CLINICAL TARGETS

Investigators have published studies on the effects of wild-type p53 gene therapy on more than 100 cell lines and tissues (Tables 1–3[15–65]). The general conclusion from these studies is that the introduction of wild-type p53 into neoplastic cells *in vitro* is lethal in most cells, if the cells are $p53^{mut}$ or $p53^{null}$. However, as Polyak et al[51] reported, some cell lines are only growth arrested. In most cases, $p53^{wt}$ neoplastic cells and normal cells were unaffected unless other factors influenced p53 function, such as human papillomavirus (HPV). Intratumoral dosing of $p53^{mut}$ or $p53^{null}$ tumor xenografts in immunocompromised mice resulted in tumor growth inhibition and often, tumor regression. In addition, two studies have demonstrated a reduction of $p53^{mut}$ mammary tumor metastases in the lungs after intravenous dosing with two different p53 vectors.[58,60] Dosing via the intrahepatic artery has also shown therapeutic benefits against $p53^{mut}$ hepatocellular carcinoma in Buffalo rats,[65] while dosing via the intrahepatic vein was not effective in SV40 Tag transgenic mice with liver tumors.[60]

Numerous investigators have demonstrated expression of wild-type p53 after introduction of DNA into cells using adenovirus, retrovirus, herpes simplex virus, liposomal, and plasmid vectors. All tissue-types tested so far have been permissive for exogenous p53 expression. Typical effects include changes in cell morphology, cell cycle arrest, and increased apoptosis or differentiation. Endogenous gene expression is often affected as well. Induction of p21 (WAF1/CIP1) has been reported after expression of exogenous p53 in prostate tumor cells,[19] ovarian tumor cells,[22] colorectal tumor cells,[22,51] head and neck tumor cells,[44] mammary tumor cells,[18,22] and mammary tumor xenografts.[60] Induction of MDM-2 has been reported in mammary[18] and medulloblastoma cells,[67] and *gadd45* expression was induced in ovarian tumor cells.[68]

Harris et al[16] correlated the percentage of tumor cells transduced by a β-gal adenovirus with the antiproliferative effects of a p53 adenovirus (Fig 1). In $p53^{null}$ and $p53^{mut}$ cells, they found a strong positive correlation between the degree of p53-induced growth inhibition and the rate of adenovirus transduction. In contrast, cell lines expressing normal levels of wild-type p53 were minimally affected by p53 transduction, independent of the adenovirus transduction rate.

Nielsen et al[15] found that the ability of a β-gal adenovirus to transduce three mammary tumor lines *in vitro* was predictive of the *in vivo* efficacy of a p53 adenovirus against tumor xenografts. At the same adenovirus concentrations, MDA-MB-468 cells had a slightly higher transduction rate than MDA-MB-231 cells, while MDA-MB-435 cells were resistant to adenovirus transduction. Intratumoral dosing of MDA-MB-468 and -231 xenografts resulted in significant growth inhibition and regression, while MDA-MB-435 xenografts were completely unaffected. By contrast, systemic dosing of MDA-MB-435 xenografts with a liposomal formulation of the p53 gene caused growth inhibition, regression, and reduced lung metastases.[60] These results strongly suggest that the lack of MDA-MB-435 tumor response in Nielsen et al[15] was not due to an inability of p53 to inhibit the growth and metastasis of MDA-MB-435 tumors, but rather, was due to the low adenovirus transduction efficiency of this cell line.

The α_v integrins have been implicated as cellular elements required for efficient internalization of type 2, 3, and 4 adenoviruses.[69–71] It is likely that α_v integrins perform the same role for type 5 adenovirus. Wickham et al[70] observed 5–10-fold higher internalization of a recombinant type 5 adenovirus in cells transfected with $\alpha_v\beta_5$ compared with cells lacking α_v expression or transfected with $\alpha_v\beta_3$. The human embryonic kidney 293 cells used for production of E1-deleted adenoviruses express $\alpha_v\beta_1$, but not $\alpha_v\beta_3$ integrins.[72] FACS analysis demonstrated that MDA-MB-231 and MDA-MB-435 cells both express roughly equivalent levels of all these integrin family molecules.[15] Therefore, the lack of adenovirus transduction in the MDA-MB-435 line is not due to a deficiency in integrin expression. It is possible that MDA-MB-435 cells are deficient in the cellular receptor required for adenovirus binding or that some other component required for viral binding, internalization, and gene expression is defective. Only recently, has a putative adenovirus cellular receptor (CAR1) been identified.[73] Future investigations with CAR1 should elucidate the resistance mechanism in MDA-MB-435 cells.

Prior to the identification of CAR1,[73] Seth et al[17] measured the number of adenovirus-binding sites on three mammary tumor cell lines (MDA-MB-231, -453, and MCF-7) and normal bone marrow cells. Mammary tumor cells had 1–2 × 10^3 high affinity and 5–8 × 10^5 low affinity binding sites for type 5 adenovirus. By contrast, adenovirus binding sites were undetectable on bone marrow cells. Binding site quantification was predictive of transduction rates by β-gal adenovirus with 100% of mammary cells transduced at 100 PFU/cell, but no bone marrow cells transduced at 500 PFU/cell. Colony formation by MDA-MB-231 cells was reduced 55% by 1 PFU/cell of p53 adenovirus and 100% by 10

Table 1. Efficacy of p53 Gene Therapy Against Tumor cells *in vitro*

Vector	Cell lines	ED_{50} values for effects of p53 gene therapy on cell proliferation (P) or colony formation (C)	Reference
Breast cancer			
Ad	MDA-MB-231, -468, -435	NS, NS, >50 CIU/cell (P)	15
Ad	MDA-MB-231, -468, SK-BR-3, BT-549, T-47D, HBL-100, MCF-7	12, 3, 16, 2, 3, 99, >100 CIU/cell (P)	16
Ad	MDA-MB-231, -453, MCF-7	1 PFU/cell (C), NS, NS; Apop	17
Ad	MDA-MB-231, -453, -157, MCF-7, 184B5, MCF10	0.4, 0.7, 0.3, 30, 5, 6 PFU/cell (P); Apop	18
Ad	MCF-7	No effect at 20 PFU/cell (P)	19
Ad	MCF-7	Decr at 200 PFU/cell (C)	20
Ad	MDA-MB-468	2 PFU/cell (P)	21
Ad	SkBr3, MCF-7	Decr (P); Apop; greater combined efficacy with Dox, Mito, not Vinor (SkBr3)	22
Rtv	MDA-MB-468, BT549	Decr (C)	23
Ovarian cancer			
Ad	SK-OV-3, Caov-3, Caov-4, PA-1	24, >100, >100, >100 CIU/cell (P)	16
Ad	SK-OV-3	Decr (P), sensitized to radiation	24
Ad	SK-OV-3	110 CIU/cell (P), decr (C)	25
Ad	2774	10 μM (P)	26
Ad	SK-OV-3	10 PFU/cell (P)	27
Ad	SK-OV-3	Decr (P & C)	27
Ad	SK-OV-3	Decr (P)	22
Cervical cancer			
Ad	C33A, HT3, HeLa, C4-I, MS751, ME180, CaSki, SiHa	10, 53, 71, 97, 77, 138, 55, 37, PFU/cell (P); Apop	28
Ad	HeLa	80 CIU/cell (P)	16
Ad	HeLa	Decr (P)	29
Ad	HeLa	Decr (C)	20
Prostate cancer			
Ad	C4-2 (LNCaP), DU-145, PC-3	Decr (P)	30
Ad	LNCaP, DU-145, DuPro-1	Decr (P); Apop	19
Ad	Mouse 148-1PA	Decr (P)	31
Ad	Tsu-Pr1	Decr (P); Apop	32
Lung cancer			
Ad	H358, Calu-6, H661, H596, H23, H322, H460, MRC-9, A549, WI-38	2, 6, 7, 9, 3, 36, 81, 79, 79, 56 CIU/cell (P)	16
Ad	H358	0.17 PFU/cell (P)	18
Ad	H23	Sensitized to CDDP	33
Ad	H69, H596	4, 10 PFU/cell (P)	21
Ad	H226Br, H358, H322, H460	Decr (P)	34
Ad	H1299	Decr (P)	35
Ad	H358	Decr (P), sensitized to CDDP; Apop	36
Rtv	H226Br, H358	Decr (P)	37
Rtv	H358, H322, R460	Decr (P), decr (P), no effect	38
Rtv	H322, WT225	Decr (P), no effect (P)	39
Head and neck cancer			
Ad	Tu-138, MDA 686-LN	Decr (P)	40
Ad	TR146	Decr (P); Apop	41
Ad	Tu-138, Tu-177, MDA 686-LN, MDA 886	Decr (P)	40
Ad	Tu-138, Tu-177, MDA 686-LN, MDA 886	Decr (P)	42
Ad	Tu-138, MDA 686-LN	Decr (P); Apop	43
Ad	CNE-1, CNE-2Z	Decr (P and C); Apop	44
Nervous system cancer			
Ad	G55, G59, G112, G122, G124, U87 MG	6, 72, 1, 1, 1, 6 CIU/cell (P); Apop	45
Ad	G112, SK-N-MC, SN-N-SH	1, 2, 16 CIU/cell (P)	16
Ad	U-251 MG, T-98 G, U-87 MG, U-373 MG, U-138 MG, A-172, LG, EFC-2, D54 MG	Decr (P); Apop	46
Ad	Rat 9L	Decr (P)	47
Ad	T98G	Decr (P), sensitized to CDDP; Apop	38
Rtv	A673	Decr (C)	48

continued

Table 1. Continued

Vector	Cell lines	ED_{50} values for effects of p53 gene therapy on cell proliferation (P) or colony formation (C)	Reference
Bladder cancer			
Ad	HT-1376, 5637, J82, FHs 738B1	>100, 23, 40, >100 CIU/cell (P)	16
Colorectal cancer			
Ad	EB, Colo 320D, DLD-1, Colo 205, WiDr, SW480, SW837, RKO	46, 26, 12, >100, 47, 53, 52, >100 CIU/cell (P)	16
Ad	SW480	Decr (P), sensitized to radiation	49
Ad	DLD-1	7 CIU/cell (P)	21
Ad	SW620, KM12L4	Decr (P), Apop	50
Ad	SW480	Decr (P)	22
Ad	DLD-1, HCT116	Apop; Arrest	51
Liver cancer			
Ad	Hep 3B, HLE, HLF, SK-HEP-1, Hep G2	5, 1, 1, 86, 84 CIU/cell (P)	16
Ad	Hep 3B, Hep G2	7, 60 PFU/cell (P)	21
Skin cancer			
Ad	SK-MEL-24, Mouse B16	Decr (P); Apop	52
Muscle cancer			
Ad	A673, SK-UT-1	7, 5 CIU/cell (P)	16
Bone cancer			
Ad	Saos-2	2 CIU/cell (P)	16
Ad	Saos-2	Decr (P); Apop	53
Ad	Saos-2	Decr (P); Apop	54
Ad	Saos-2	1 PFU/cell (P)	21
Rtv	Saos-2	Decr (P, C)	6
Lymphomas/leukemias			
Vac	HL-60	Decr (P); incr Apop and differentiation	55
Ad	JB6	Decr (C)	20
Ad	K-562	>100 PFU/cell	21
Rtv	Be-13	Decr (C, P)	56
Normal tissue			
Ad	$CD34^+$ bone marrow	1000 PFU/cell (C)	17
Ad	Fibroblast	No effect (P)	42
Ad	Fibroblast	No effect at 20 PFU/cell (P)	19
Ad	Fibroblast	No effect (P)	26
Ad	Fibroblast	Variable decr (P)	27
Ad	Fibroblast	30–35% decr (P & C) at 50 PFU/cell	44
Ad	Mammary epithelium (NMEC)	100 PFU/cell (P)	18
Ad	Rat astrocyte	No effect (P)	47
Ad	Rat newborn neurons	Apop	57
Ad	Bronchial epithelium	No effect at 100 PFU/cell (P)	35

Ad = Adenovirus, Rtv = Retrovirus, Lip = Liposomal DNA, Vac = vaccinia virus. All cells are human unless otherwise indicated. P = cell proliferation inhibited. C = colony formation inhibited. ED_{50} = dose which caused a 50% reduction in P or C. Apop = apoptosis documented. NS = not studied. CDDP = cisplatin. Dox = Doxorubicin/Adriamycin. Mito = Mitomycin C.

PFU/cell. p53 adenovirus had no effect on colony formation by bone marrow cells at concentrations up to 100 PFU/cell. At a concentration of 1000 PFU/cell, bone marrow colonies were reduced by 50%, however at this very high adenovirus concentration cytotoxicity might be mediated by mechanisms other than p53 expression.

Viral oncoproteins, such as HPV E6, can inactivate wild-type p53 and hence, induce a mutant-p53 phenotype in cells lacking alterations in the p53 gene. HPV infections are especially prevalent in cervical tumors, with a growing number of reports in head and neck tumors.[74] Hamada et al[28] examined the efficacy of a p53 adenovirus in eight cervical cancer cell lines. Two of the lines expressed mutant p53, while the other six had wild-type p53 inactivated by HPV. Proliferation of all cell lines was inhibited by p53 adenovirus with a range of ED_{50} values from 9 to 149 PFU/cell. The p53 adenovirus induced apoptosis in infected cells, as well as, reversing tumorigenicity *in vivo*. In addition, p53 adenovirus treatment of established tumor xenografts from four cervical lines dramatically reduced tumor growth.

Combination therapy

Investigations into the efficacy of p53 gene therapy in combination with other therapeutics are only now start-

Table 2. p53 Protein Status of Cells Used in Gene Therapy Studies

Tissue	p53 status	Cell lines
Mammary	Mutant	BT-549, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, SK-BR-3, T-47D
	Null	MDA-MB-157
	Wild-type	184B5, HBL-100, MCF-7, MCF-10
Ovarian	Mutant	2774, Caov-4
	Null	SK-OV-3, Caov-3
	Wild-type	PA-1
Cervical	Mutant	C33A, HT3
	WT/HPV	C4-I, CaSki, HeLa, ME180, MS751, SiHa
Prostate	Mutant	DU-145
	Null	Mouse 148-1PA, Tsu-Pr1, PC-3
	Wild-type	PC-82, LNCaP (silent mutation)
Lung	Mutant	H23, H226Br, H322, H596, H661
	Null	Calu-6, H69, H358, H1299
	Wild-type	A549, MRC-9, WI-38, WT225, H460
Head/Neck	Mutant	TR146, Tu-138, Tu-177, CNE-1, CNE-2Z
	Null	SqCC/Y1
	Wild-type	MDA 886-LN, MDA 886,
Nervous Sys	Mutant	A172, Daoy, G59, G112, G122, G124, LG, SL, T98G, U138MG, U251MG, U373MG, Del4A
	Null	A673, SK-N-MC
	Wild-type	EFO-2, D54 MG, G85, SN-N-SH, U87 MG
Bladder	Mutant	5637, J82, HT-1376
	Wild-type	FHs 738B1
	Unknown	Mouse MBT-2
Colorectal	Mutant	Colo 205, Colo 320D, DLD-1, SW480, SW620, SW837, WiDr, KM12L4
	Null	EB
	Wild-type	RKO, HCT116
Liver	Mutant	HLE, HLF, McA-RH7777
	Null	Hep 3B
	Wild-type	Hep G2, SK-HEP-1
Skin	Unknown	SK-MEL-24, Mouse B16
Muscle	Mutant	SK-UT-1
	Null	A673
Bone	Null	Saos-2
Leuk/Lymph	Null	K-562, U-937, HL-60, Be-13
	Unknown	JB6

Primary references for the p53 status of tumor cell lines can be downloaded at the Internet address ftp://ftp.ebi.ac.uk/pub/databases/p53 or can be found in the articles listed at the end of this review.

ing to appear. Fujiwara et al[36] demonstrated additive benefits in $p53^{null}$ H358 lung cancer when p53 gene therapy was combined with the DNA damaging agent, cisplatin. H358 cells cultured with cisplatin for 24 hours before transduction with p53 adenovirus had a significantly lower rate of proliferation than cells treated with either agent alone. When cells were transduced with p53 adenovirus 24 hours before exposure to cisplatin, there was a dose-dependent cisplatin effect. H358 cells or spheroids exposed to both agents exhibited greater apoptosis, as evidenced by DNA fragmentation. An additive efficacy of both agents, with enhanced apoptotic death, was also demonstrated *in vivo*. However, it should be noted that 1) the subcutaneous H358 xenografts were only 5 mm^3 at the beginning of the experiment and control tumors only reached a volume of 30 mm^3 on the last day of tumor measurements; 2) only 12 days elapsed from the start of dosing to the end of the two studies; and 3) the first cisplatin dose (3 mg/kg 3 ×) causes an average body weight loss of 26% in nude mice by day 7 and the 6 × dose would have been lethal (L.L.N., personal observation). Stronger evidence came from Nguyen et al.[61] In this study, $p53^{null}$ H1299 lung tumor xenografts were dosed with intraperitoneal cisplatin before, concurrent with, or after intratumoral p53 adenovirus. The most effective dosing regime was 5 mg/kg cisplatin given 2 days before three doses of 5×10^9 viral particles/day of p53 adenovirus, with the adenovirus doses administered 2 days apart. A second cycle of therapy produced increased efficacy over a single cycle.

Gjerset et al[33] demonstrated increased sensitivity to cisplatin cytotoxicity in $p53^{mut}$ T98G glioblastoma and $p53^{mut}$ H23 small cell lung carcinoma cells transduced with p53 expression vectors 1 or 2 days before cisplatin exposure. Cell death mediated by apoptosis was significantly increased versus p53-transduced cells, when T98G cells were transduced by 100 PFU/cell of p53 adenovirus 2 days before exposure to 30 μM cisplatin. Additive efficacy was also seen for p53 and γ-irradiation. Yang et al[73] used $p53^{mut}$ SW480 colorectal tumor cells transfected with an IPTG-inducible p53 plasmid construct to evaluate the combined efficacies of p53 with 5-fluorouracil (5-FU; 0–20 μM), p53 with topotecan (0–10 μM), and p53 with γ-irradiation (0–400 cGy for 1 hour). All three agents displayed dose-dependent effects on cell cytotoxicity which were enhanced by concurrent expression of wild-type p53. DNA fragmentation was elevated in cells exposed to both p53 and 5-FU. Furthermore, the potentiation of 5-FU cytotoxicity by p53 was greatest when cells were exposed to both agents simultaneously. Blagosklonny and El-Deiry[74] reported increased cell killing in $p53^{mut}$ SkBr3 mammary tumor cells when transduction with p53 Ad was followed 8 hours later by doxorubicin or mitomycin C, but not by vincristine. Greater combined efficacy was not observed in $p53^{wt}$ MCF-7 mammary tumor cells for any of the three drugs.

Additional studies on the ability of wild-type p53 to sensitize tumor cells to irradiation have been reported for colorectal and ovarian tumor cells.[24,49] SW620 colorectal tumor cells ($p53^{mut}$) were transduced with 50 PFU/cell p53 adenovirus 48 hours before irradiation with 2 or 4 Gy.[49] Cell survival was reduced by 50–66% compared to mock- or vector-infected irradiated cells, and this reduction was mediated by apoptotic cell death. Efficacy was also highest in SW620 xenografts pretreated with three consecutive doses of p53 adenovirus before irradiation with 5 Gy. Again, apoptosis was most evident in tumors treated with both agents. Similar, although not as dramatic, results have been reported for $p53^{null}$

Table 8. Efficacy of p53 Gene Therapy Against Tumor Xenografts

Cell line	Vector	Route	Dose *in vivo*	Efficacy	Reference
Breast cancer					
MDA-MB-231	Ad	T/P	2×10^9 CIU, 10×	95% growth inhibition, 80% tumor-free, Apop	58
MDA-MB-468	Ad	T/P	2×10^9 CIU, 10×	80% growth inhibition, 10% tumor-free, Apop	58
MDA-MB-435	Ad	T/P	2×10^9 CIU, 10×	No efficacy	58
MDA-MB-231	Ad	T/P	$2–4 \times 10^9$ CIU, 10×	79–88% growth inhibition; 60–80% decr lung metastases	15
MDA-MB-231	Ad	i.v.	4×10^9 CIU, 5×	71% decr no. lung metas, decr metastases size	15
MDA-MB-435	Lip	i.v.	16/12 μg DNA, 2×	75% growth inhib	59
MDA-MB-435	Lip	i.v.	35 μg DNA, 6×	Growth inhib, 53% regressed, 67% lung metastasis-free	60
MCF-7	Lip	i.v.	16/12 μg DNA, 3×	40% growth inhibition	59
Ovarian cancer					
SK-OV-3	Ad	T/P	1×10^9 PFU, 1×	Sensitized to irradiation	24
SK-OV-3	Ad	e.v., i.p.	2×10^9 CIU, 6×	Incr survival; marginal incr survival	25
			2×10^9 CIU, 6×	Marginal incr survival	
Cervical cancer					
C33A	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 86% growth inhib & 29% tumor-free	28
HT3	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 86% growth inhib & 71% tumor-free	28
HeLa	Ad	e.v.	NA	100% tumor suppression	28
MS751	Ad	e.v., T/P	5×10^9 PFU, 6×	100% tumor suppression; 88% growth inhib & 14% tumor-free	28
SiHa	Ad	e.v., T/P	5×10^9 PFU, 1×, 3×, 6×	100% tumor suppression; 62% growth inhib, 92% growth inhib; 95% growth inhib & 20% tumor-free	28
Prostate cancer					
04-2 (LNCaP)	Ad	T/P	1×10^9 PFU, 6×, 8×	Growth inhib, 88% tumor-free; Apop	30
DU-145	Ad	e.v.	NA	100% tumor suppression	30
PC-3	Ad	e.v.	NA	100% tumor suppression	30
Mouse 148-1PA	Ad	T/P	5×10^9 PFU, 1×	21% growth inhibition	31
Tsu-Pr1	Ad	e.v.	NA	90% tumor suppression	32
Lung cancer					
H1299	Ad	T/P	5×10^9 PN/cell,	Incr efficacy in combination with CDDP (1, 2)	61
H69	Ad	T/P	2×10^8 PFU, 1×, 8×	Incr survival	21
H226Br	Ad	i.t.	5×10^7 PFU, 2×	78% growth inhibition, 75% tumor-free vs 20–30% of controls	34
H358	Ad	T/P	2×10^7 PFU, 9×	Growth inhibition; Additive efficacy with CDDP (3)	36
H226Br	Rtv	i.t.	8×10^8 PFU, 3×	64% growth inhib, 63% tumor-free vs. 25% of controls	37
Head and neck cancer					
Tu-138	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
Tu-177	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
MDA 686-LN	Ad	T/P	1×10^8 PFU, 1×	100% tumor suppression	42
MDA 886	Ad	T/P	1×10^8 PFU, 1×	67% tumor suppression	42
MDA 686-LN	Ad	T/P	1×10^7 PFU, 1×	Apoptosis in tumors	43
Tu-138	Ad	T/P	1×10^9 PFU, 1×	97% growth inhibition	62
Tu-177	Ad	T/P	1×10^9 PFU, 1×	98% growth inhibition	62
Nervous system cancer					
G122	Ad	e.v, T/P	7×10^8 CIU, 3×	100% tumor suppression	45
Rat 9L	Ad	T/P	1×10^7 PFU, 1×	40% growth inhibition	47
A573	Rtv	e.v.	NA	Tumor suppression	48
Bladder cancer					
Mouse MBT-2	Ad	i.e., i.p.	1.5×10^9 PFU, 1×	No efficacy	63
Colorectal cancer					
DLD-1	Ad	T/P	1×10^9 CIU, 5×	Growth inhibition, incr survival	16
SW620	Ad	T/P	2.5×10^9 PFU, 3×	Growth inhibition, incr apoptosis after irradiation	49
SW620, KM12L4	Ad	T/P	3.3×10^9 PFU, 3×	Growth inhibition, incr apoptosis	50
Liver cancer					
McA-RH7777	Ad	IHA	???	Growth inhibition	64
Skin cancer					
SK-MEL-24	Ad	T/P	2×10^9 PFU, 1X	Growth inhibition	62
Mouse B16	Ad	T/P	2×10^9 PFU, 1X	Growth inhibition	62

continued

Table 3. Continued

Cell lines	Vector	Route	Dose *in vivo*	Efficacy	Reference
Bone cancer					
Saos-2	Ad	e.v.	NA	100% tumor suppression	21
Saos-2	Rtv	e.v.	NA	100% tumor suppression	6
Normal tissue					
Rat liver	Ad	PV	5×10^8 PFU, 1X	No effect on liver regeneration after hepatectomy	65

Ad = adenovirus, Lip = liposomal DNA, Rtv = retrovirus, T/P = intra-/peritumoral, i.v. = intravenous, i.p. = intraperitoneal, i.t. = intratracheal, IHA = intrahepatic artery, PV = hepatic portal vein, e.v. = ex vivo, i.s. = intravesicular, NA = not applicable, CDDP = cisplatin at: 1) 5 mg/kg, i.p., 1 ×; 2) 1.67 mg/kg, 3 ×; 3) 3 mg/kg, i.p., 3 times. Apop = apoptosis-documented. All cells are human unless otherwise indicated.

SK-OV-3 ovarian tumor cells.[24] Cells transduced with p53 adenovirus and subsequently irradiated with 2 or 4 Gy had approximately 8–30% lower survival than mock- or vector-infected irradiated cells. Subcutaneous tumor xenografts were treated once with p53 adenovirus or the appropriate controls, then irradiated with 4 Gy/day on 3 consecutive days. This dosing regime was repeated 1 week later. Combination therapy with p53 and irradiation had significantly increased efficacy against tumor xenografts and cured 45% of the mice.

The preliminary conclusion, which can be gleaned from these seven studies,[23,24,33,36,49,61,75] is that p53 gene therapy combined with DNA-damaging agents has additional efficacy over p53 gene therapy alone. Further, no observations of antagonistic interactions between p53 gene therapy and more traditional anticancer therapeutic agents have been reported. In particular, cisplatin pretreatment might sensitize tumors to subsequent p53 gene therapy. The converse situation, p53 pretreatment of tumors, has not proven as effective in sensitizing tumors to cisplatin. However, this result may be due to physical factors and not a true deficiency in p53 effects. When mice are given intraperitoneal doses of cisplatin, the drug reaches most, if not all, tumor cells via the blood supply. On the other hand, the *in vivo* experiments reported to date have used an adenovirus vector and direct injection into the xenograft and surrounding tissues to deliver the p53 gene. The architecture of this tumor system limits diffusion of the adenovirus, and thereby, limits the number of infectable target cells which come in contact with the adenovirus. In very small tumors, a sizeable fraction of the tumor cells may be transduced by one dose of p53 adenovirus, but this situation is the exception rather than the rule. This was clearly demonstrated in Nielsen et al,[15] where fractionated doses of p53 adenovirus had increased antitumor efficacy over fewer, higher doses.

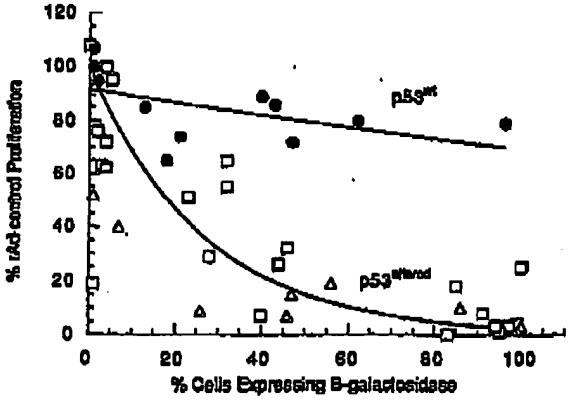

Figure 1. p53-specific growth inhibition as a function of adenovirus transduction. p53-specific antiproliferative effects of p53 adenovirus were measured at 30 CIU/cell in a 72 hours [^{3}H]thymidine incorporation assay and normalized for the effects of an adenovirus vector control. Adenovirus transduction was measured as the percentage of cells expressing β-galactosidase 24 hours after infection with a β-gal adenovirus. Open symbols represent individual p53-altered cell lines (squares, $p53^{mut}$; triangles, $p53^{null}$). Closed symbols represent cell lines expressing endogenous wild-type p53. Data are presented as means of replicate experiments. (Reprinted from Ref. 16).

In a eighth study on combination therapy, cell differentiation, not cell death was the end result. Ehringer et al[76] transfected $p53^{null}$ U-937 leukemia cells with a temperature-sensitive murine p53 mutant which converted to wild-type conformation at 32°C. Expression of wild-type, but not mutant, p53 slowed cell proliferation, caused cells to accumulate in the G1 phase of the cell cycle, and induced apoptosis. Somewhat paradoxically, expression of wild-type p53 sensitized cells to differentiation mediated by vitamin D3 and this effect "overrode" the apoptosis pathway.

Clinical efficacy

The potential for toxicity in normal tissue, caused by the expression of exogenous p53, is an issue of concern for any clinical protocol. Preclinical data are encouraging on this issue. Investigators have observed little or no detrimental effects on normal fibroblasts, bronchial epithelium, mammary epithelial cells, bone marrow cells, rat astrocyte cells, and rat liver at concentrations of p53 which are highly effective at killing neoplastic cells.[17–19,26,27,35,42,47] The most common route of p53 administration to tumor xenografts has been through intra- and peritumoral injection. No gross necrosis or other abnormalities of tissues surrounding injection sites has been reported in the literature or observed in our laboratories (L.N. and D.M.). One cautionary report has been published.[57] The

investigators transduced newborn Sprague–Dawley rat neurons with recombinant adenoviruses *in vitro* and found dose-dependent p53-specific toxicity, in addition to the vector-specific toxicity discussed below.

To date, only one clinical study has been published in which vector transduction of target tissues was confirmed. Roth et al[77] transduced lung tumors using a p53 retrovirus introduced into patients via fiberoptic bronchoscope or percutaneous needle with radiologic guidance. Nine male patients with a history of primary non-small cell lung carcinoma (NSCLC) and recurrent or metastastic tumors were enrolled in the phase I study. All nine patients had mutations in the p53 gene. Vector sequences were detected in eight of the treated tumors. In addition, six out of seven evaluated tumors showed evidence of increased apoptosis. Tumors regressed in three of the seven patients and no toxicity due to p53 therapy was observed. The results of this promising phase I human trial support cautious optimism for the future of p53 gene therapy of cancer.

FUTURE CHALLENGES

Vector toxicology

The most significant elements in the emerging toxicological profile for recombinant adenoviruses are the localized inflammatory response at the site of administration and the interference with normal hepatocyte functioning caused by high intravascular concentrations of virus. Zhang et al[35] reported on the toxicology of B1-deleted p53 adenovirus in mouse lungs. Intratracheal adenovirus at 10^7 to 10^{10} PFU/mouse was administered to Balb/c mice, and lungs were harvested 1, 3, 6, and 12 days after inoculation. No pathological changes were observed at the 10^7 and 10^8 PFU dose levels. However, at the 10^9 and 10^{10} PFU doses there was a mild inflammation characterized by perivascular and peribronchial infiltration of mononuclear cells.

At least three groups have reported changes in hepatocyte function at very high doses of E1-deleted adenovirus.[78–80] Cultured mouse hepatocytes were 100% transduced by recombinant adenovirus at 100 PFU/cell without any toxic effects, however at virus concentrations ≥ 1000 PFU/cell cytotoxicity was observed.[78] C57Bl/6 mice dosed with 1×10^{10} viral particles via the hepatic portal vein did not exhibit toxic effects. However, infusion of 7×10^{10} viral particles was lethal in most of the mice, due to liver necrosis. Drazan et al[79] studied liver function in male Brown Norway rats after *ex vivo* infusion of β-gal adenovirus or empty vector via the hepatic portal vein and subsequent liver transplantation into syngeneic hosts. Transduction with 50 PFU/cell β-gal adenovirus resulted in ascites and lethality due to liver necrosis in three out of four rats. Liver necrosis was absent in livers infused with vehicle or empty vector. Yang et al[80] infused 1×10^{10} PFU retrograde into the billiary tracts of female CBA and athymic nude mice. CBA mice infused with a β-gal adenovirus developed liver pathology characterized by ballooning degeneration of hepatocytes and cell death, followed by increased hepatic mitoses and some lymphocytic infiltration. Nude mice had similar hepatocyte abnormalities, but no lymphocyte infiltration and hepatic mitoses.

At least one group has reported on the effects of intravenous p53 liposomes on organ histopathology.[59] The first p53 liposome dose contained 16 μg of DNA with 400 nmol of liposome (= 1:1 ratio of 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine and dioleoyl phosphatidylethanolamine). Ten days later a second injection of 12 μg of DNA with 400 nmol of liposome was given. Athymic nude mouse organs were harvested 14 days after the last injection. No pathological changes were observed in heart, lung, liver, pancreas, spleen, kidney, intestine, or skin. Also, blood chemistry was unchanged by p53 liposomal treatment.

Immune response to adenoviruses

Prevailing theory holds that adenovirus infection generates a rapid inflammatory and cytolytic response mediated by cytotoxic T cells in hosts with fully functional immune systems.[81] This T-cell response is stimulated by adenovirus antigens produced in host cells and presented in conjunction with major histocompatibility complex moieties on the cell surface. Neutralizing antibodies specific for cells transduced by adenovirus are produced later in the immune response and are believed responsible for the reduced ability to re-infect host cells with adenovirus after initial inoculations.

One proposed solution to the adenovirus immune system problem is to create vectors in which most of the late viral genes are deleted. A cautionary note for this strategy can be found in Adesanya et al,[82] where injection of either bioactive or UV-inactivated adenoviruses into rat salivary gland caused a sharp reduction in saliva production, in other words, inflammatory cell infiltration and tissue damage. This occurred despite the lack of gene transcription from the UV-irradiated virus. Adenoviral and liposomal vectors both cause some tumor growth inhibition without p53 expression in mouse xenograft models. The mechanism of this antitumor effect is unclear at present. It may be partially mediated by nonspecific immune cells such as NK cells,[54] however other mechanisms are also likely to play a role.

Bystander effects

There is some evidence that p53 might exert some of its antitumor activity through inhibition of angiogenesis.[59,83] $p53^{null}$ fibroblasts from Li-Fraumeni patients secreted reduced levels of thrombospondin-1, an angiogenesis inhibitor, compared to early passage $p53^{wt}$ fibroblasts.[78] An anti-thrombospondin-1 antibody restored the migration of bFGF-stimulated capillary endothelial cells which was induced by $p53^{wt}$ fibroblast-conditioned medium, implying a role for thrombospondin-1 in angiogenesis suppression. Restoration of wild-type p53 function in $p53^{null}$ fibroblasts resulted in higher levels of thrombospondin-1 and lower angiogenic activity in conditioned medium. Other evidence for a role of p53 in

suppressing angiogenesis was reported by Xu et al.[59] MDA-MB-435 mammary tumor xenografts in nude mice showed significant growth inhibition and reduced blood vessel density when mice were dosed with intravenous p53 liposomes. This result is especially surprising given the low tumor transduction rate of 5% in this experiment.

CONCLUDING REMARKS

The preclinical studies reviewed above have clearly demonstrated the feasibility of p53 gene therapy for cancer in a variety of models. Expression of p53 in cancer cells lacking this tumor suppressor can lead to cell apoptosis or cycle arrest, and delivery of p53 may also inhibit the angiogenesis required for tumor growth. Although retroviral vectors were used initially to demonstrate the utility of p53 reintroduction, alternative delivery strategies are required for successful p53 gene therapy in the clinical setting. Adenoviral vectors provide an efficient method for locoregional delivery and transient overexpression of p53, and this strategy may enable effective p53 gene therapy for the treatment of certain malignancies. Ongoing clinical investigations will provide critical information on the safety and efficacy of this approach. However, significant advances in current gene delivery technology are needed to increase the efficiency of gene transfer to metastatic disease.

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